Method of inhibiting prostate cancer cell proliferation

Field of the Invention

The present invention relates to a method of inhibiting proliferation of prostate cancer cells, such as in a method of treating prostate cancer. In particular, the invention relates to a method of inhibiting proliferation of androgen-independent prostate cancer cells.

Background of the invention

Prostate cancer occurs frequently in men, is currently the second most common cause of cancer-related death and incidence is growing. Prostatectomy is useful in the treatment of patients with cancer confined to the prostate. Androgen ablation therapy is used in cases where cancer cells still require androgens for growth but have spread beyond the prostate. To date, however, there is no effective treatment for metastatic Androgen-Independent Prostate Cancer (AIPC).

Our understanding of the aetiology of prostate cancer is limited and unlike certain other cancers, little progress has been made in elucidating its cause. Efforts have been made to identify genes responsible for familial prostate cancer. At least seven chromosomal loci have been reported, however the genes responsible for prostate cancer in all these loci have not yet been identified. Although an inherited genetic predisposition occurs in only 5-10% of cases, it is possible that identification of germline mutations may shed light on sporadic cases as both forms share the same histopathological features. The majority of researchers have focused on somatic defects in sporadic prostate cancer. Classical cytogenetic studies are difficult to apply to solid tumours and so far no consistent chromosomal changes have been observed. Although comparative genome hybridisation and loss of heterozygosity analysis have

shown both gain and loss of genomic DNA, the majority of genes involved are still unknown. Oncogenes and tumour suppressor genes known to be associated with other malignancies have a remarkably low frequency of mutation or deletion in prostate cancer. Using technologies that compare the steady-state mRNA levels between normal and cancerous prostate, a list of genes have been revealed to be either over or underexpressed in prostate cancer tissue or cell-lines. Although proteomics and tissue array approaches are now being used, relatively few genes have yet been verified to be differentially expressed in a reasonable number of specimens at the protein level. Direct evidence for the importance of these differentially expressed genes in prostate cancer initiation or progression is lacking. As a result, although progress is rapid, the application of this new knowledge in controlling mortality and morbidity from prostate cancer is slow at present.

Emerging evidence from epidemiological studies indicates a strong association between prostate cancer risk and total fat intake (Kolonel et al., 1999 J. Natl Cancer Inst. 91: 414), although the biochemical link between dietary lipids and genesis of prostate cancer remains unclear. Previous studies have demonstrated that both cyclooxygenase (COX) and lipoxygenase (LOX) products of arachidonic acid metabolism, the prostaglandins (PG), and hydroxyeicosatetraenoic acids (HETEs) respectively, contribute to formation and/or progression of prostate cancer. They are implicated in promotion of tumour cell proliferation, motility, invasion and metastasis, and induction of angiogenesis both *in vitro* and in animal models. Interestingly, arachidonic acid levels are lower in malignant than benign (BPH) prostate tissue while PG and HETE synthesis from labelled arachidonic acid is significantly increased. However, the activity of arachidonic acid mobilising enzymes phospholipase A_2 (PLA_2) and fatty acyl-CoA lysophosphatidylcholine acyltransferase, are also increased, suggesting an increased flux of arachidonic acid through the COX and LOX pathways.

PLA_2 constitutes a large and diverse family of enzymes that catalyse the hydrolysis of membrane phospholipids at the *sn*-2 position to release fatty acids and lysophospholipids. PLA_2 enzymes are classified according to their source and their cellular location (i.e secreted PLA_2 enzymes ($sPLA_2$s) or cytosolic PLA_2 enzymes ($cPLA_2$s)). A review of the classification and characterisation of the expanding superfamily of PLA_2 enzymes had been published by Six and Dennis (2000) *Biochim. Biophys. Acta* 1488:1-19.

$sPLA_2$-IIA is elevated in prostate cancer (Graff et al., 2001, Clin. Cancer Res. 7: 3857-3861; Jiang et al., 2002, Am. J. Pathol. 160: 667-671) and enhanced $sPLA_2$-IIA expression has been inversely related to 5-year patient survival (Graff et al., 2001). In addition, the chromosomal location of several $sPLA_2$ genes including $sPLA_2$-IIA (1p35-ter), overlaps with one prostate cancer susceptibility locus CAPB (Nwosu et al., 2001, Human Mol. Genet. 10: 2313-2318). To date, however, there has been no evidence to show that $sPLA_2$-IIA is involved in prostate tumorigenesis.

Summary of the invention

We have now shown by immunohistochemistry in patients following androgen ablation therapy, that Type IIA secreted phospholipase A_2 ($sPLA_2$-IIA) is elevated in androgen-independent tumour cells relative to benign glands, while cytosolic phospholipase A_2-α ($cPLA_2$-α) levels are unchanged.

We have also found that treatment of prostate cancer cells with $sPLA_2$-IIA potently increases proliferation of the cells, and that this proliferative effect is blocked by the addition of selective inhibitors of both $sPLA_2$-IIA and $cPLA_2$-α enzymes.

This provides evidence for the first time of the direct role of PLA_2 in the proliferation of prostate cancer cells and identifies this class of enzymes as an important therapeutic target for the treatment of prostate cancer.

Accordingly, in a first aspect the present invention provides a method of inhibiting or reducing the proliferation of prostate cancer cells, the method comprising administering to the cells a PLA_2 inhibitor.

In a second aspect the present invention provides a method for the treatment of prostate cancer, the method comprising administering to a subject in need thereof a PLA_2 inhibitor.

In a preferred embodiment of the first and second aspects, the prostate cancer cells are androgen independent prostate cancer (AIPC) cells.

In accordance with the first and second aspects of the present invention, the PLA_2 inhibitor may inhibit any PLA_2 enzyme. Preferably, the inhibitor inhibits an enzyme selected from group 1B, IIA, IID, IIE, IIF, III, IV, V, and X PLA_2 enzymes.

In one embodiment, the inhibitor is a $cPLA_2$-α inhibitor. For example, the inhibitor may be pyrrolidine-1, a substituted pyrimidine as described in WO 00/27824, a 9,10-dihydro-9,10-ethanoanthracene derivative as described in WO 99/15493, an azalomycin inhibitor as described in JP12119292, an arylsulfonamide as described in WO 98/25893, an indole-2-carboxylic acid derivative as described in WO 98/05637, an indole derivative as described in WO 98/08818, WO 99/43651, WO 99/43654 or WO 99/43672, a 3-sulfanyl-propane-1,2-diol derivative as described in JP12038380, a heterocyclic compound such as those described in WO 00/34254 or an oxazolidinedione or thiazolidinedione derivative as described in WO 97/05135 or WO 98/33797.

In another embodiment, the inhibitor is an sPLA$_2$-IIA inhibitor. For example, the inhibitor may be a benzoic acid as described in JP8325154, a 6-aza-spiro-[4,5]-decane derivative as described in JP9110835, amide derivatives of fatty acids such as those described in WO 00/00220 and WO 95/19959, (arylsulfonamidophnoxy) benzoic acids such as those described in WO 97/35567, fatty acid derivatives such as those described in WO 97/38966, tetronic acids such as those described in JP0045740, dinitrogen heterocyclic compounds such as those described in WO 98/05332, oxadiazine and thioxadiazine derivatives such as those described in WO 00/71118, sulfonylaminopyrazoles such as those described in WO 98/24437, (Indol-3-yl)acetamide inhibitors described in EP 839806, EP 950657, EP 952149 and WO 00/07590, (indol-3-yl) oxoacetamides such as those described in EP 675110, WO 98/37069, WO 99/59999, WO 99/51605, WO 00/07591, WO 00/37358 and WO 00/00201, or derivatives such as those described in WO 99/21545, WO 99/21546 and WO 99/21559.

In a particularly preferred embodiment the inhibitor is a conformationally constrained molecule derived from a peptide consisting essentially of amino acid residues 70 to 74 of a human sPLA$_2$-IIA protein, or the equivalent residues in other sPLA$_2$-IIA proteins.

Preferably, the conformationally constrained molecule is a peptide, more preferably a cyclic peptide.

In a preferred embodiment, the conformationally constrained peptide is a cyclic peptide of the following formula:

A1-A2-A3-A4-A5

in which

A1 is F or Y or W or 2Nap

A2 is L or I

A3 is S or T

A4 is F or Y or W or 2Nap
A5 is R or K.

In a further preferred embodiment of the present invention, the peptide is selected from the group consisting of cFLSYK, cFLSYR and c(2NapA)LS(2NapA)R.

When used herein the term "cFLSYK" means "cyclic FLSYK", "cFLSYR" means "cyclic FLSYR" and "c(2NapA)LS(2NapA)R" means "cyclic (2Nap)LS (2Nap)R". The term "2NapA" is an abbreviation for 2-naphthylalanine.

In a further preferred embodiment, the peptide comprises D-amino acids and has a sequence which corresponds to the reverse sequence of a peptide according to the first aspect of the present invention.

In a preferred embodiment of the present invention, the method involves administration of a $sPLA_2$-IIA inhibitor in conjunction with a $cPLA_2$-α inhibitor.

In yet a further aspect the present invention provides the use of a PLA_2 inhibitor in the manufacture of a medicament for the treatment of prostate cancer.

In yet a further aspect the present invention provides the use of a conformationally constrained molecule derived from a peptide consisting essentially of amino acid residues 70 to 74 of a human $sPLA_2$-IIA protein, or the equivalent residues in other $sPLA_2$-IIA proteins, in the manufacture of a medicament for the treatment of prostate cancer.

In yet another aspect the present invention provides a method for detecting prostate cancer or a metastasis thereof in a subject, said method comprising:

determining the level of PLA_2 mRNA expressed in a test sample from said subject; and

comparing the level of PLA_2 mRNA determined at (i) to the level of PLA_2.mRNA expressed in a comparable sample from a healthy or normal individual,

wherein a level of PLA_2 mRNA at (i) that is enhanced in the test sample relative to the comparable sample from the normal or healthy individual is indicative of the presence of a cancer cell in said subject.

In yet another aspect the present invention provides a method for detecting prostate cancer or a metastases thereof in a subject, said method comprising:

determining the level of a PLA_2 polypeptide in a test sample from said subject; and

comparing the level of PLA_2 polypeptide determined at (i) to the level of said PLA_2 polypeptide in a comparable sample from a healthy or normal individual,

wherein a level of said PLA_2 polypeptide at (i) that is enhanced in the test sample relative to the comparable sample from the normal or healthy individual is indicative of the presence of a cancer cell in said subject.

In yet another aspect the present invention provides a method of assessing the predisposition of a subject to prostate cancer, the method comprising the step of determining the presence of a polymorphism or an epigenetic change in a PLA_2 gene of the subject.

Brief description of the Figures

Figure 1. Effect of androgen ablation therapy on $sPLA_2$-IIA protein expression. Prostate tissue from patients following radical prostatectomy either with (N = 25) or without (N=50) prior androgen ablation therapy were examined for $sPLA_2$-IIA protein expression by immunohistochemistry as described in Materials and Methods. A. Adjacent sections from a patient without androgen-ablation therapy showing normal glands. B. Adjacent sections from a patient following androgen ablation therapy showing both normal and neoplastic tissue. Left panel stained with haematoxilin and eosin, right panel stained with anti-$sPLA_2$-IIA antibody. Magnification, x 40. C. $sPLA_2$-IIA expression in normal (open boxes) or neoplastic (closed boxes) tissue from patients with (+) or without (-) androgen ablation therapy was graded on a 3-point score, (1; 0 - 33% tissue positive, 2; 33 - 66% tissue positive and 3; >66% tissue positive). *; $P < 0.05$ vs no androgen ablation therapy treated benign glands by Chi-Square analysis.

Figure 2. Effect of exogenous addition of $sPLA_2$-IIA on prostate cell growth. LNCaP cells were grown in RPMI with 5% FCS and treated for 72 hours in media containing (A) increasing concentrations of $sPLA_2$-IIA alone (closed bars) or increasing concentrations of the activity-dead $sPLA_2$-IIA mutant $H_{48}Q$. (B) a fixed concentration of $sPLA_2$-IIA and increasing concentrations of the $sPLA_2$-IIA inhibitor cFLSYR. Cell number relative to untreated control cells was determined by the MTS assay as described in Materials and Methods. Data are Mean ± SD of quadruplicate determinations normalised to 100% for untreated cells. OD_{495} of unstimulated cells was 0.29 ±0.06 for $sPLA_2$-IIA and 0.24 ± 0.03 for the $sPLA_2$-IIA mutant experiments in panel A and 0.24 ± 0.03 for panel B. * $p<0.05$ vs untreated control by one-way ANOVA. Data are representative of three separate experiments. (C). LNCaP cells were grown as above and stimulated for 72 hours with $sPLA_2$-IIA (1 nM) in the absence (dotted lines) and presence (solid lines) of the $sPLA_2$-IIA inhibitor cFLSYR (100 nM). Cells

($1x10^6$) were assayed by flow cytometry following treatment with propidium iodide as described in Materials and Methods. Statistical analysis was performed on 10,000 events per sample. Data are representative of three separate experiments.

Figure 3. Effect of inhibition of $cPLA_2$-α on $sPLA_2$-IIA-dependent proliferation. LNCaP cells were grown in RPMI containing 5% FCS and stimulated for 72 hours with $sPLA_2$-IIA (1 nM) in the presence (hatched bars) and absence (closed bar) of increasing concentrations of the $cPLA_2$-α-selective inhibitor pyrrolidine-1. Cell number was determined by the MTS assay as described in Materials and Methods. Data are mean ± SD of quadruplicate determinations expressed as percentages relative to untreated control cells (100%) (open bar). OD_{495} of untreated cells was 0.33 ± 0.03. In the presence of pyrrolidine-1 (5 uM) alone, OD_{495} was 0.38 ± 0.04 * $p<0.05$ vs untreated control by one-way ANOVA.

Figure 4. Effect of $sPLA_2$-IIA inhibition on unstimulated prostate cancer cells. (A) The endogenous expression of $sPLA_2$-IIA mRNA was evaluated by RT-PCR in three unstimulated prostate cancer cell lines (LNCaP, DU145 and PC-3) grown in RPMI containing 5% FCS. HPRT was used to as a positive control for RNA integrity and loading. (B) Cells were grown in RPMI with 5% FCS were then treated for 72 hours in the presence and absence of the $sPLA_2$-IIA inhibitor c(2Nap)LS(2Nap)R and cell number determined by MTS assay as described in Materials and Methods. Data are mean ± SD of quadruplicate determinations expressed as percentages relative to untreated control cells (100%). LNCaP, closed bars, OD_{495} untreated cells 0.50 ± 0.03; DU145, open bars, OD_{495} untreated cells 0.73 ± 0.05; PC-3, hatched bars, OD_{495} untreated cells, 0.66 ±0.01. * $p<0.05$ vs untreated control by one-way ANOVA.

Detailed description of the invention

Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art (e.g. in cell culture, molecular genetics, nucleic acid chemistry, hybridization techniques and biochemistry). Standard techniques are used for molecular, genetic and biochemical methods (see generally, Sambrook et al., Molecular Cloning: A Laboratory Manual, 2nd ed. (1989) Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y. and Ausubel et al., Short Protocols in Molecular Biology (1999) 4th Ed, John Wiley & Sons, Inc. - and the full version entitled Current Protocols in Molecular Biology, which are incorporated herein by reference) and chemical methods.

Throughout this specification the word "comprise", or variations such as "comprises" or "comprising", will be understood to imply the inclusion of a stated element, integer or step, or group of elements, integers or steps, but not the exclusion of any other element, integer or step, or group of elements, integers or steps.

PLA_2 Enzymes

The methods of the present invention encompass targeting any PLA_2 enzyme. A review of the classification and characterisation of the expanding superfamily of PLA_2 enzymes had been published by Six and Dennis (2000) *Biochim. Biophys. Acta* 1488:1-19, and the present methods contemplate targeting all of these enzymes.

In preferred embodiments of the present invention, the PLA_2 enzyme is $sPLA_2$-IIA or $cPLA_2$-α. Preferably, the PLA_2 enzyme is a human enzyme.

The nucleic acid and protein sequences for human sPLA$_2$-IIA are shown in SEQ ID NOs:1 and 3 respectively. The nucleic acid and protein sequences for human cPLA$_2$-α are shown in SEQ ID NOs:2 and 4 respectively.

PLA$_2$ Inhibitors

Protein or Peptide inhibitors

In one embodiment, peptidyl PLA$_2$ inhibitors are chemically or recombinantly synthesized as oligopeptides (approximately 10-25 amino acids in length) derived from the a PLA$_2$ sequence (for example, SEQ ID NO:3 or 4). Alternatively, PLA$_2$ fragments are produced by digestion of native or recombinantly produced PLA$_2$ by, for example, using a protease, e.g., trypsin, thermolysin, chymotrypsin, or pepsin. Computer analysis (using commercially available software, e.g. MacVector, Omega, PCGene, Molecular Simulation, Inc.) is used to identify proteolytic cleavage sites. The proteolytic or synthetic fragments can comprise as many amino acid residues as are necessary to partially or completely inhibit PLA$_2$ function. Preferred fragments will comprise at least 5, 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95, 100 or more amino acids in length.

Protein or peptide inhibitors may also be dominant-negative mutants of PLA$_2$. The term "dominant-negative mutant" refers to a PLA$_2$ polypeptide that has been mutated from its natural state and that interacts with a protein that PLA$_2$ normally interacts with thereby preventing endogenous native PLA$_2$ from forming the interaction.

Anti-PLA$_2$ Antibodies

The term "antibody" as used in this invention includes intact molecules as well as fragments thereof, such as Fab, F(ab')2, and Fv which are capable of

binding an epitopic determinant of PLA_2. These antibody fragments retain some ability to selectively bind with its antigen and are defined as follows:

(1) Fab, the fragment which contains a monovalent antigen-binding fragment of an antibody molecule can be produced by digestion of whole antibody with the enzyme papain to yield an intact light chain and a portion of one heavy chain;

(2) Fab', the fragment of an antibody molecule can be obtained by treating whole antibody with pepsin, followed by reduction, to yield an intact light chain and a portion of the heavy chain; two Fab' fragments are obtained per antibody molecule;

(3) (Fab')2, the fragment of the antibody that can be obtained by treating whole antibody with the enzyme pepsin without subsequent reduction; F(ab)2 is a dimer of two Fab' fragments held together by two disulfide bonds;

(4) Fv, defined as a genetically engineered fragment containing the variable region of the light chain and the variable region of the heavy chain expressed as two chains; and

(5) Single chain antibody ("SCA"), defined as a genetically engineered molecule containing the variable region of the light chain, the variable region of the heavy chain, linked by a suitable polypeptide linker as a genetically fused single chain molecule.

Methods of making these fragments are known in the art. (See for example, Harlow and Lane, Antibodies: A Laboratory Manual, Cold Spring Harbor Laboratory, New York (1988), incorporated herein by reference).

Antibodies of the present invention can be prepared using intact PLA_2 or fragments thereof as the immunizing antigen. A peptide used to immunize an animal can be derived from translated cDNA or chemical synthesis and is purified and conjugated to a carrier protein, if desired. Such commonly used carriers which are chemically coupled to the peptide include keyhole limpet hemocyanin (KLH), thyroglobulin, bovine serum albumin (BSA), and tetanus toxoid. The coupled peptide may then be used to immunize the animal (e.g., a mouse or a rabbit).

If desired, polyclonal antibodies can be further purified, for example, by binding to and elution from a matrix to which the peptide to which the antibodies were raised is bound. Those of skill in the art will know of various techniques common in the immunology arts for purification and/or concentration of polyclonal antibodies, as well as monoclonal antibodies (See for example, Coligan, et al., Unit 9, Current Protocols in Immunology, Wiley Interscience, 1991, incorporated by reference).

Monoclonal antibodies may be prepared using any technique which provides for the production of antibody molecules by continuous cell lines in culture, such as, for example, the hybridoma technique, the human B-cell hybridoma technique, and the EBV-hybridoma technique (Kohler et al. Nature 256, 495-497, 1975; Kozbor et al., J. Immunol. Methods 81, 31-42, 1985; Cote et al., Proc. Natl. Acad. Sci. USA 80, 2026-2030, 1983; Cole et al., Mol. Cell Biol. 62, 109-120, 1984).

Methods known in the art allow antibodies exhibiting binding for PLA_2 to be identified and isolated from antibody expression libraries. For example, a method for the identification and isolation of an antibody binding domain which exhibits binding to PLA_2 is the bacterio-phage a vector system. This vector system has been used to express a combinatorial library of Fab fragments from the mouse antibody repertoire in Escherichia coli (Huse, et al., Science,

246:1275-1281, 1989) and from the human antibody repertoire (Mullinax, et al., Proc. Nat. Acad. Sci., 87:8095-8099, 1990). This methodology can also be applied to hybridoma cell lines expressing monoclonal antibodies with binding for a preselected ligand. Hybridomas which secrete a desired monoclonal antibody can be produced in various ways using techniques well understood by those having ordinary skill in the art and will not be repeated here. Details of these techniques are described in such references as Monoclonal Antibodies-Hybridomas: A New Dimension in Biological Analysis, Edited by Roger H. Kennett, et al., Plenum Press, 1980; and U.S. 4,172,124, incorporated by reference.

In addition, methods of producing chimeric antibody molecules with various combinations of "humanized" antibodies are known in the art and include combining murine variable regions with human constant regions (Cabily, et al. Proc. Natl. Acad. Sci. USA, 81:3273, 1984), or by grafting the murine-antibody complementarity determining regions (CDRs) onto the human framework (Riechmann, et al., Nature 332:323, 1988).

Antisense compounds

The term "antisense compounds" encompasses DNA or RNA molecules that are complementary to at least a portion of a PLA_2 mRNA molecule (Izant and Weintraub, Cell 36:1007-15, 1984; Izant and Weintraub, Science 229(4711):345-52, 1985) and capable of interfering with a post-transcriptional event such as mRNA translation. Antisense oligomers complementary to at least about 15 contiguous nucleotides of PLA_2-encoding mRNA are preferred, since they are easily synthesized and are less likely to cause problems than larger molecules when introduced into the target PLA_2-producing cell. The use of antisense methods is well known in the art (Marcus-Sakura, Anal. Biochem. 172: 289, 1988). Preferred antisense nucleic acid will comprise a nucleotide

sequence that is complementary to at least 15 contiguous nucleotides of a sequence encoding the amino acid sequence set forth in SEQ ID NO:3 or 4.

Catalytic nucleic acids

The term catalytic nucleic acid refers to a DNA molecule or DNA-containing molecule (also known in the art as a "DNAzyme") or an RNA or RNA-containing molecule (also known as a "ribozyme") which specifically recognizes a distinct substrate and catalyzes the chemical modification of this substrate. The nucleic acid bases in the catalytic nucleic acid can be bases A, C, G, T and U, as well as derivatives thereof. Derivatives of these bases are well known in the art.

Typically, the catalytic nucleic acid contains an antisense sequence for specific recognition of a target nucleic acid, and a nucleic acid cleaving enzymatic activity (also referred to herein as the "catalytic domain"). To achieve specificity, preferred ribozymes and DNAzymes will comprise a nucleotide sequence that is complementary to at least about 12-15 contiguous nucleotides of a sequence encoding the amino acid sequence set forth in SEQ ID NO:3 or 4.

The types of ribozymes that are particularly useful in this invention are the hammerhead ribozyme (Haseloff and Gerlach 1988, Perriman et al., 1992) and the hairpin ribozyme (Shippy et al., 1999).

The ribozymes of this invention and DNA encoding the ribozymes can be chemically synthesized using methods well known in the art. The ribozymes can also be prepared from a DNA molecule (that upon transcription, yields an RNA molecule) operably linked to an RNA polymerase promoter, e.g., the promoter for T7 RNA polymerase or SP6 RNA polymerase. Accordingly, also provided by this invention is a nucleic acid molecule, i.e., DNA or cDNA, coding

for the ribozymes of this invention. When the vector also contains an RNA polymerase promoter operably linked to the DNA molecule, the ribozyme can be produced in vitro upon incubation with RNA polymerase and nucleotides. In a separate embodiment, the DNA can be inserted into an expression cassette or transcription cassette. After synthesis, the RNA molecule can be modified by ligation to a DNA molecule having the ability to stabilize the ribozyme and make it resistant to RNase. Alternatively, the ribozyme can be modified to the phosphothio analog for use in liposome delivery systems. This modification also renders the ribozyme resistant to endonuclease activity.

RNA inhibitors

dsRNA is particularly useful for specifically inhibiting the production of a particular protein. Although not wishing to be limited by theory, Dougherty and Parks (1995) have provided a model for the mechanism by which dsRNA can be used to reduce protein production. This model has recently been modified and expanded by Waterhouse et al. (1998). This technology relies on the presence of dsRNA molecules that contain a sequence that is essentially identical to the mRNA of the gene of interest, in this case an mRNA encoding a PLA_2 protein. Conveniently, the dsRNA can be produced in a single open reading frame in a recombinant vector or host cell, where the sense and anti-sense sequences are flanked by an unrelated sequence which enables the sense and anti-sense sequences to hybridize to form the dsRNA molecule with the unrelated sequence forming a loop structure. The design and production of suitable dsRNA molecules targeted agasint PLA_2 is well within the capacity of a person skilled in the art, particularly considering Dougherty and Parks (1995), Waterhouse et al. (1998), WO 99/32619, WO 99/53050, WO 99/49029, and WO 01/34815.

As used herein, the terms "small interfering RNA", and "RNAi" refer to homologous double stranded RNA (dsRNA) that specifically targets a gene

product, thereby resulting in a null or hypomorphic phenotype. Specifically, the dsRNA comprises two short nucleotide sequences derived from the target RNA encoding PLA_2 and having self-complementarity such that they can anneal, and interfere with expression of a target gene, presumably at the post-transcriptional level. RNAi molecules are described by Fire et al., Nature 391, 806-811, 1998, and reviewed by Sharp, Genes & Development, 13, 139-141, 1999).

Small molecule inhibitors

Small molecule inhibitors that regulate PLA_2 are described in Lehr (2001) Expert Opin. Ther. Patents (2001) 11 (7):1123-1136, and Hansford *et al* (2003) Chem. Biochem. 4:181-185.

Peptides and peptide analogues

In preferred embodiments, the methods of the present invention involve the administration of conformationally constrained molecules derived from a peptide consisting essentially of amino acid residues 70 to 74 of a human $sPLA_2$-IIA protein, or the equivalent residues in other $sPLA_2$-IIA proteins.

In general, reference to amino acid residues 70 to 74 of the human $sPLA_2$-IIA protein is taken to include reference to the equivalent residues in other $sPLA_2$-IIA proteins, such as orthologues of human $sPLA_2$-IIA.

The term "conformationally constrained molecules" means conformationally constrained peptides and conformationally constrained peptide analogues and derivatives.

Thus the conformationally constrained molecules according to the present invention include conformationally constrained peptides consisting essentially

of residues 70 to 74 of the human $sPLA_2$-IIA protein, and analogues and derivatives thereof.

The term "analogues" refers to molecules having a chemically analogous structure to the naturally occurring alpha-amino acids present as residues 70 to 74 of the human $sPLA_2$-IIA protein. Examples include molecules containing *gem*-diaminoalkyl groups or alklylmalonyl groups.

The term "derivatives" includes alpha amino acids wherein one or more side groups found in the naturally occurring alpha-amino acids present as residues 70 to 74 of human $sPLA_2$-IIA protein have been modified. Thus, for example the naturally-occurring amino acids present in residues 70 to 74 of the human $sPLA_2$-IIA protein may be replaced with a variety of uncoded or modified amino acids such as the corresponding D-amino acid or N-methyl amino acid. Other modifications include substitution of hydroxyl, thiol, amino and carboxyl functional groups with chemically similar groups.

The present invention encompasses the use of conformationally constrained peptidomimetics of the biologically active human $sPLA_2$-IIA peptide (amino acid residues 70 to), i.e. analogues and derivatives which mimic the activity of said peptide and are therefore capable of inhibiting the $sPLA_2$-IIA dependent proliferation of prostate cancer cells. These peptidomimetics are preferably substantially similar in both three-dimensional shape and biological activity to the specific $sPLA_2$-IIA peptides described herein. Substantial similarity means that the geometric relationship of groups in the peptide that react with the $sPLA_2$-IIA enzyme is preserved and at the same time, that the peptidomimetic will inhibit the $sPLA_2$-IIA dependent proliferation of prostate cancer cells.

A peptidomimetic is a molecule that mimics the biological activity of a peptide but is no longer peptidic in chemical nature. By strict definition, a peptidomimetic is a molecule that no longer contains any peptide bonds (that

is, amide bonds between amino acids). However, the term peptide mimetic is sometimes used to describe molecules that are no longer completely peptidic in nature, such as pseudo-peptides, semi-peptides and peptoids. Whether completely or partially non-peptide, peptidomimetics for use in the methods of the invention provide a spatial arrangement of reactive chemical moieties that closely resembles the three-dimensional arrangement of active groups in the peptide on which the peptidomimetic is based. As a result of this similar active-site geometry, the peptidomimetic has effects on biological systems which are similar to the biological activity of the peptide.

There are clear advantages for using a mimetic of a given peptide rather than the peptide itself, because peptides commonly exhibit two undesirable properties: (1) poor bioavailability; and (2) short duration of action. Peptide mimetics offer an obvious route around these two major obstacles, since the molecules concerned are small enough to be both orally active and have a long duration of action. There are also considerable cost savings and improved patient compliance associated with peptide mimetics, since they can be administered orally compared with parenteral administration for peptides. Furthermore, peptide mimetics are much cheaper to produce than peptides.

Suitable peptidomimetics based on residues 70 to 74 of human $sPLA_2$-IIA peptides and having similar biological activities, and therefore similar therapeutic utilities, can be developed using readily available techniques. Thus, for example, peptide bonds can be replaced by non-peptide bonds that allow the peptidomimetic to adopt a similar structure, and therefore biological activity, to the original peptide. Further modifications can also be made by replacing chemical groups of the amino acids with other chemical groups of similar structure. The development of peptidomimetics derived from $sPLA_2$-IIA peptides based on residues 70 to 74 of human $sPLA_2$-IIA can be aided by determining the tertiary structure of the original peptide by NMR spectroscopy, crystallography and/or computer-aided molecular modelling. These techniques

aid in the development of analogues/derivatives of higher potency and/or greater bioavailability and/or greater stability than the original peptide (Dean, 1994, BioEssays, 16: 683-687; Cohen and Shatzmiller, 1993, J. Mol. Graph., 11: 166-173; Wiley and Rich, 1993, Med. Res. Rev., 13: 327-384; Moore, 1994, Trends Pharmacol. Sci., 15: 124-129; Hruby, 1993, Biopolymers, 33: 1073-1082; Bugg et al., 1993, Sci. Am., 269: 92-98.

Information on the structure of an $sPLA_2$-IIA peptide consisting essentially of residues 70 to 74 of human $sPLA_2$-IIA can be used to search three-dimensional databases to identify molecules having a similar structure, using programs such as MACCS-3D and ISIS/3D (Molecular Design Ltd., San Leandro, CA), ChemDBS-3D (Chemical Design Ltd., Oxford, U.K.), and Sybyl/3DB Unity (Tripos Associates, St. Louis, MO).

Databases of chemical structures are available from a number of sources including Cambridge Crystallographic Data Centre (Cambridge, U.K.), Chemical Abstracts Service (Columbus, OH), and ACD-3D (Molecular Design Ltd).

De novo design programs include Ludi (Accelrys), Leapfrog (Tripos Associates) and Aladdin (Daylight Chemical Information Systems, Irvine, CA).

Those skilled in the art will recognize that the design of a mimetic may require slight structural alteration or adjustment of a chemical structure designed or identified using these databases.

Peptide derivatives and peptidomimetic compounds based on amino acid residues 70 to 74 of human $sPLA_2$-IIA can be tested to determine whether they are capable of inhibiting $sPLA_2$-IIA dependent proliferation of prostate cancer cells using the assay described herein. Preferred peptide derivatives and peptidomimetics have at least 90%, preferably at least the same anti-

proliferative activity toward prostate cancer cells as cFLSYR. It is also preferred that peptide derivatives and peptidomimetics specifically inhibit $sPLA_2$-IIA.

The molecules, such as peptides, used in the methods of the present invention are conformationally constrained. Conformational constraint refers to the stability and preferred conformation of the three-dimensional shape assumed by a peptide. Conformational constraints include local constraints, involving restricting the conformational mobility of a single residue in a peptide; regional constraints, involving restricting the conformational mobility of a group of residues, which residues may form some secondary structural unit; and global constraints, involving the entire peptide structure.

The active conformation of a peptide may be stabilized by a covalent modification, such as cyclization or by incorporation of gamma-lactam or other types of bridges. For example, side chains can be cyclized to the backbone so as create a L-gamma-lactam moiety on each side of the interaction site. See, generally, Hruby *et al.*, 1992, "Applications of Synthetic Peptides," in Synthetic Peptides: A User's Guide: 259-345 (W. H. Freeman & Co.). Cyclization also can be achieved, for example, by formation of cystine bridges, coupling of amino and carboxy terminal groups of respective terminal amino acids, or coupling of the amino group of a Lys residue or a related homolog with a carboxy group of Asp, Glu or a related homolog. Coupling of the alpha-amino group of a polypeptide with the epsilon-amino group of a lysine residue, using iodoacetic anhydride, can be also undertaken. See, for example, Wood and Wetzel, 1992, *Int'l J. Peptide Protein Res.* 39: 533-39.

Another approach described in US 5,891,418 is to include a metal-ion complexing backbone in the peptide structure. Typically, the preferred metal-peptide backbone is based on the requisite number of particular coordinating groups required by the coordination sphere of a given complexing metal ion. In

general, most of the metal ions that may prove useful have a coordination number of four to six. The nature of the coordinating groups in the peptide chain includes nitrogen atoms with amine, amide, imidazole, or guanidino functionalities; sulfur atoms of thiols or disulfides; and oxygen atoms of hydroxy, phenolic, carbonyl, or carboxyl functionalities. In addition, the peptide chain or individual amino acids can be chemically altered to include a coordinating group, such as for example oxime, hydrazino, sulfhydryl, phosphate, cyano, pyridino, piperidino, or morpholino.

A further approach approach is to use bifunctional crosslinkers, such as N-succinimidyl 3-(2 pyridyldithio) propionate, succinimidyl 6-[3-(2 pyridyldithio) propionamido] hexanoate, and sulfosuccinimidyl 6-[3-(2 pyridyldithio) propionamido]hexanoate (see US Patent 5,580,853).

Techniques for chemically synthesising the peptides and derivatives described above are described in the above references and also reviewed by Borgia and Fields, 2000, *TibTech* 18: 243-251 and described in detail in the references contained therein.

Diagnostic methods

The present invention also encompasses nucleic acid-based methods and protein-based methods for diagnosing prostate cancer in humans and other mammals.

As used herein, the term "diagnosis", and variants thereof, such as, but not limited to "diagnose", "diagnosed" or "diagnosing" shall not be limited to a primary diagnosis of a clinical state, however should be taken to include any primary diagnosis or prognosis of a clinical state. For example, the "diagnostic assay" formats described herein are equally relevant to assessing the remission of a patient, or monitoring disease recurrence, or tumor recurrence,

such as following surgery or chemotherapy, or determining the appearance of metastases of a primary tumor. All such uses of the assays described herein are encompassed by the present invention.

Accordingly, the level of PLA_2 expression, at either the RNA level or the protein level, can be used to diagnose prostate cancer, or as a prognostic to monitor the progress of prostate cancer.

Preferred nucleic acid-based diagnostic assays rely upon the detection or relative quantification of RNA levels in samples using probes of at least about 20 nucleotides in length that hybridize specifically to RNA encoding PLA_2, or alternatively, amplify cDNA from RNA encoding PLA_2. Conveniently, any hybridization assay format can be used to detect PLA_2-encoding RNA in samples, such as, for example, high-throughput screening using microarray technology, or conventional northern hybridization or reverse transcription polymerase chain reaction (i.e. RT-PCR). In situ localization can also be employed using histology specimens.

Suitable diagnostic immunoassays utilize antibodies, including monoclonal and polyclonal antibodies, or a Fab fragment, F(ab')2 fragment, or scFv fragment, that binds to a unique peptide region comprising at least about 5-10 contiguous amino acid residues of PLA_2.

The present invention further encompasses any synthetic or recombinant peptides, or antibodies suitable for use in the assays described herein.

In preferred embodiments of these diagnostic methods the PLA_2 is $sPLA_2$-IIA or $cPLA_2$-α.

In another aspect, the invention relates to methods of diagnosing for predisposition to prostate cancer.

Accordingly, in one aspect the present invention provides a method of assessing the predisposition of a subject to prostate cancer, the method comprising the step of determining the presence of a polymorphism or an epigenetic change in a PLA_2 gene of the subject.

In one embodiment, the polymorphism is a PLA_2 polymorphism already identified in a public or private database such as the NCBI database or the Celera database.

The present invention encompasses nucleic-acid based methods and protein-based methods for diagnosing susceptibility to prostate cancer.

The polymorphism in the PLA_2 gene may be a point mutation (i.e. a single nucleotide polymorphism (SNP)), deletion and/or insertion. Such a polymorphism may be detected by isolating and sequencing DNA fragments from the PLA_2 gene or otherwise by isolating mRNA from the individual and synthesising DNA therefrom (e.g. by RT-PCR) for sequencing. Polymorphisms may also be detected by hybridisation using discriminating oligonucleotide probes or by amplification procedures using discriminating oligonucleotide primers. Suitable methods may involve Southern analysis of genomic DNA; direct mutation analysis by restriction enzyme digestion; Northern analysis of RNA; denaturing high pressure liquid chromatography (DHPLC); gene isolation and sequencing; hybridization of an allele-specific oligonucleotide with amplified gene products; exon trapping, single base extension (SBE); or analysis of a PLA_2 protein.

In one embodiment the epigenetic change is aberrant methylation in a PLA_2 gene of the subject or insertion of an endogenous retroviral promoter or transposable element promoter close to a PLA_2 gene of the subject.

Methods of screening for therapeutic agents

Also encompassed by the present invention are methods of identifying therapeutic agents useful for the treatment of prostate cancer.

Accordingly, in a first aspect the present invention provides a method of screening for a compound that reduces or inhibits the proliferation of prostate cells, the method comprising determining the activity of PLA_2 in the presence and absence of a candidate compound, wherein reduced PLA_2 activity in the presence of the compound indicates that the compound reduces or inhibits the proliferation of prostate cells.

In another aspect, the present invention provides a method of screening for a compound that reduces or inhibits the proliferation of prostate cells, the method comprising determining the expression levels of PLA_2 in the presence and absence of a candidate compound, wherein reduced PLA_2 expression in the presence of the compound indicates that the compound reduces or inhibits the proliferation of prostate cells.

In a further embodiment of this aspect, the method involves exposing a translation system capable of expressing PLA_2 to a candidate compound and comparing the levels of expression of PLA_2 in the presence of the compound to the levels achieved under similar conditions but in the absence of the compound. The translation system may be a cell-free translation system. Alternatively, the translation system may comprise eukaryotic or prokaryotic cells.

In a further aspect the present invention provides a method of screening for a compound that reduces or inhibits the proliferation of prostate cells, the method comprising determining the ability of a candidate compound to modulate the binding of PLA_2 to a PLA_2 substrate, wherein an altered level of binding of PLA_2 to the substrate in the presence of the compound indicates that the compound reduces or inhibits the proliferation of prostate cells.

In preferred embodiments of these screening methods the PLA_2 is $sPLA_2$-IIA or $cPLA_2$-α.

Therapeutic methods

We have shown that administration of exogenous $sPLA_2$-IIA to prostate cells stimulates cell proliferation. We have also shown that administration of PLA_2 inhibitors inhibits $sPLA_2$-IIA mediated cell proliferation. Consequently, PLA_2 inhibitors can be used to inhibit or reduce prostate cell proliferation in cells, particularly in cells with elevated sPLA-IIA activity such as prostate cancer cells.

The PLA_2 inhibitors can be used therapeutically for prostate cancers, particularly AIPCs. The term "therapeutically" or as used herein denotes both prophylactic as well as therapeutic administration. Thus, PLA_2 inhibitors can be administered to high-risk patients in order to lessen the likelihood and/or severity of prostate cancer or administered to patients already evidencing prostate cancer.

The peptides, analogues and small molecule inhibitors described above may preferably be combined with various components to produce compositions. Preferably the compositions are combined with a pharmaceutically acceptable carrier or diluent to produce a pharmaceutical composition (which may be for human or animal use). Suitable carriers and diluents include isotonic saline solutions, for example phosphate-buffered saline, water, dry powders and micelles. The composition may be administered by any means known in the art. Modes of delivery include, but are not limited to, direct injection, topical delivery (e.g. by atomised nasal delivery or nasal drops) or oral delivery. Accordingly, the composition may be formulated, *inter alia*, for topical, parenteral, intramuscular, intravenous, subcutaneous, intraocular, oral or transdermal administration.

Typically, each peptide or analogue or derivative thereof may be administered at a dose of from 0.01 to 30 mg/kg body weight, preferably from 0.1 to 10 mg/kg, more preferably from 0.1 to 1 mg/kg body weight.

The routes of administration and dosages described are intended only as a guide since a skilled practitioner will be able to determine readily the optimum route of administration and dosage for any particular patient and condition.

The present invention will now be described further with reference to the following examples which are intended to be illustrative only and non-limiting.

EXAMPLES

Materials and Methods

Cell Lines and Culture. The LNCaP, DU145 and PC3 cell lines were purchased from American Type Culture Collection (Rockville, MD). All cell cultures were maintained in RPMI 1640 supplemented with 5% FBS for LNCaP and 10% FBS for DU145 and PC3 at 37°C in a humidified environment of 5% CO_2. For experiments with addition of androgens, cells were cultured in RPMI 1640 without phenol red and supplemented with same concentration of charcoal stripped FBS. For experiments described in this article, we used cells between passage 30 and 45 for LNCaP, 65 and 80 for DU145 and 25 and 40 for PC3.

Reagents. $sPLA_2$-IIA was purified by immunoaffinity chromatography from conditioned media derived from Chinese hamster ovary cell line (5A2) expressing human $sPLA_2$-IIA as described (Bidgood, M.J. *et al.* J. Immunol. (2000) 165:2790-2797). A cDNA encoding the activity-dead mutant $sPLA_2$-IIA $H_{48}Q$ was constructed by oligonucleotide-mediated site-directed mutagenesis using standard molecular techniques. This cDNA was expressed in Chinese hamster ovary cells under the control of the human metallothionein promoter.

$H_{48}Q$ was purified from conditioned media derived from this cell line by immunoaffinity chromatography. Purity was verified by the presence of a single band on a silver-stained SDS gel and by amino terminal peptide sequence analysis. Enzyme activity was undetectable in the purified protein as determined by the radiolabelled *Eschericia coli* assay (Church, W.B. *et al.* (2001), J. Biol. Chem. 276:33156-33614). Both mutant protein and $sPLA_2$-IIA were quantified by ELISA (Smith, G.M. et al., Br. J. Rheumatol. (1992) 31: 175). $sPLA_2$-IIA inhibitors (Church, W.B. et al) were synthesised using Fmoc (N-(9-fluoroenyl)methoxycarbonyl) solid phase chemistry without removal of side-chain protection groups prior to cleavage from the resin. Peptides were cyclised using standard peptide synthesis activation and coupling chemistry prior to deprotection (Auspep, Melbourne). The $cPLA_2$ inhibitor pyrrolidone-1 was a kind gift from Dr Michael Gelb.

Antibodies used in these experiments included polyclonal anti-$sPLA_2$-IIa (160502, Cayman Chemicals, monoclonal anti-$sPLA_2$-IIA antibody 4A1 raised by ourselves (Smith, G.M. *et al.*), and anti-$cPLA_2$ polyclonal antibody (SC-438, Santa Cruz).

Cell growth assay. Cells were plated at 1 x 10^4 per well in 96-well plates with 0.1 ml of FBS-supplemented RPMI. After reaching 70-80% confluence (about 48 hours), the medium was changed to that containing various treatments for 3 days. After treatment, the number of viable cells was determined using the MTS assay (CellTiter 96® AQueous Assay; Promega, Madison, WI). In brief, 20 ul of MTS solution was added to each well and cells were incubated for 1 h. The absorbance at 490 nm was measured with an ELISA microplate reader. Each experiment was performed in quadruplicate and repeated at least three times.

Flow Cytometric Analysis. Cells were seeded in 25 ml flasks in conditions as described above. Following treatment, trypsinization and cell counting, LNCaP

cells ($1x10^6$) were suspended in 1 mL PBS and incubated with 0.2 mL 0.4 % Triton X-100 for 5 min at R/T in the presence of 50 μL of propidium iodide solution (50 μg/mL) and 20 μL of ribonuclease (10 mg/mL). DNA content per cell was measured by flow cytometry using an FACScalibur flow cytometer and CellQuest software (Becton Dickinson, Franklin Lakes, NJ). Statistical analysis was performed on 10,000 events per sample.

RT-PCR. Total cellular RNA was isolated from LNCaP, DU145 or PC-3 cells using the Trizol reagent (LifeTechnologies, Inc.). First-strand cDNA was synthesized from 5 μg of RNA with the cDNA preamplification system (Life Technologies, Inc.) using SuperScript II reverse transcriptase and an oligo(dT) primer. This was used as the template in standard PCR reactions using Amplitaq DNA polymerase (Perkin-Elmer Life Sciences, Boston, MA). Amplification products were analyzed on 2% TAE agarose gels made with MetaPhor agarose (FMC BioProducts, Rockland, ME) and photographed under UV illumination. DNA ladders of 25 and 100 bp (Life Technologies, Inc.) were used as size standards. Primers were designed based on the human $sPLA_2$-IIA mRNA (NM_000300.2) deposited in the GenBank database (National Center for Biotechnology Information, Bethesda, MD) and are as follows: forward: 5'-TTTGTCACCCAAGAACTCTTAC-3', reverse: 5'-GGGAGGGAGGGTATGAGA-3'.

Tissues. Normal prostate was obtained from brain-dead organ donors as described previously (Chetcuti *et al.,* (2001) Cancer Research, 61:6331-6334). Benign prostatic hyperplasia (BPH) was obtained from transurethral resection of the prostate. Cancer tissue was from radical prostatectomy. Informed consent was obtained from patients, and the study protocol was approved by the Central Sydney Area Health Service Ethics Review Committee.

Immunohistochemistry. Prostate tissues (normal, benign hyperplastic and cancer) were all fixed in 10% formaldehyde solution for <24 hours and paraffin

embedded. Tissue sections (5 μm) were incubated for 1 hour at 37°C after microwave antigen retrieval with the appropriate primary antibody diluted in 1% goat serum. Biotinylated goat anti-rabbit or mouse IgG, diluted 1/200 in 1% normal goat serum, was used as the secondary antibody. The signal was amplified using the avidin-biotin-peroxidase complex system (Vector Laboratories) and visualised using the liquid DAB substrate-chromogen system (Dako). Sections were counterstained with hematoxylin. Negative isotype and method controls were performed for each sample by substituting the primary antibody with rabbit IgG and 1% goat serum, respectively.

Image Analysis. Immunoperoxidase staining was considered positive and specific when the intensity of with the post-immune IgG clearly exceeded that observed with the preimmune IgG (isotype control) and with no IgG (method control). Specific staining was graded for the percentage of immunopositive normal or cancer epithelial cells as described (Kommoss, 1989 Anal. Quant. Cytol. Histol. 11:298-306). Briefly, the percentage of positive-stained normal, or cancerous epithelial cells per slide was stratified into three groups: group 1, 0-33%; group 2, 34-66%; and group 3, 67-100%. Zero represented no cells with specific staining.

Statistical Analysis. The Number Cruncher Statistical System (NCSS, Kaysville, UT) was used for statistical analysis. Data were analyzed by one-way ANOVA and correlation coefficient as appropriate. A two-tailed P value <0.05 was considered significant.

Example 1: $sPLA_2$-IIA is Induced in Prostate Cancer Cells and Constitutively Activated in AIPC.

We searched the database of Serial Analysis of Gene Expression (SAGE) to determine the steady state mRNA levels of individual PLA_2 enzymes in prostate cancer. The cDNA libraries used for SAGE was PR317 normal prostate and

PR317 prostate cancer, respectively (www.ncbi.nlm.nih.gov/SAGE), as both are derived from microdissected prostate tissues. We found that $sPLA_2$-IIA mRNA was 22 times higher in prostate cancer than normal prostate, whereas other members were either not expressed in the prostate libraries or unchanged in cancer. To verify the SAGE result and extend the expression analysis to androgen-independent prostate cancer (AIPC), we examined $sPLA_2$-IIA expression by immunohistochemistry in prostate cancer tissues from patients treated with androgen-ablation therapy for 3 months prior to radical prostatectomy. Cancer cells remaining in specimens following androgen-ablation therapy are regarded as being closest to AIPC, although they are confined within the prostate. Cancer specimens from patients undergoing radical prostatectomy without androgen ablation therapy served as the control. Two antibodies were used for immunohistochemistry, and both showed the same expression pattern. In the control group, (N=50), there was weak and patchy staining in benign glands (Fig. 1A and C.) adjacent to cancer cells and extensive staining in cancer cells (Fig. 1 C.). In the androgen-ablated group (N=25), benign glands lost their staining, whereas AIPC cells maintained $sPLA_2$-IIA expression (Fig. 1 B and C). We also found that the extent of $sPLA_2$-IIA staining is positively correlated with the tumour grade and post-operative PSA level (data not shown). The chromosomal location of $sPLA_2$-IIA (1p35.1-36) was also found to overlap with a prostate cancer susceptibility locus CAPB (Gibbs *et al* (1999) Am. J. Hum. Genet. 64:776-787). No difference was found in immunohistochemical staining for $cPLA_2$-α between normal and cancer cells irrespective of androgen status (data not shown).

The lack of $sPLA_2$–IIA expression in benign glands following androgen deprivation suggests that expression of $sPLA_2$–IIA gene requires androgens. To verify that, we searched the 5'- flanking region of the $sPLA_2$–IIA gene using MatInspector Release 5.3 (Genomatix), and found an androgen response element (ARE) GAGGTAAATGGTATTCTC from -546 to -527. Secondly, we treated the androgen responsive human prostate cancer cell line, LNCaP, with

various doses of androgens and measured $sPLA_2$–IIA mRNA and protein levels by RT-PCR and ELISA (data not shown). Indeed, there was an increase in the level of $sPLA_2$–IIA transcript following 1 nM androgen treatment. In contrast, we could not find an ARE within 3 kb of genomic DNA in the 5-flanking region of the $cPLA_2$-α gene. Androgen treatment had no effect on $cPLA_2$-α mRNA and protein levels (data not shown). Together, these findings indicate that $sPLA_2$-IIA, but not $cPLA_2$-α expression is normally dependent on androgens and in AIPC $sPLA_2$-IIA expression becomes androgen-independent *via* an as yet unknown mechanism.

Example 2: Oncogenic Action of $sPLA_2$–IIA in Prostate Cancer Cells.

To examine the biological relevance of $sPLA_2$-IIA to prostate cancer cell growth, we treated LNCaP cells with increasing doses of human recombinant $sPLA_2$-IIA and monitored cell growth. LNCaP is the only available prostate cancer cell line that expresses both the androgen receptor and PSA. Exogenously-added $sPLA_2$-IIA at doses as low as 1 nM, consistently and dose-dependently stimulated LNCaP cell growth as measured by the 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethylphenyl)-2-(4-sulphophenyl)-2H-tetrazolium (MTS) assay (Fig. 2A). In contrast addition of an activity-dead $sPLA_2$-IIA mutant protein $H_{48}Q$, showed no stimulation relative to untreated cells (Fig. 2A) indicating that enzyme activity is essential for the growth-stimulatory effect.

We have previously shown that human $sPLA_2$-IIA is dose-dependently inhibited by a pentapeptide sequence comprising residues 70-74 of the native $sPLA_2$-IIA protein (^{70}FLSYK^{74}) (Tseng, A., *et al.,* (1996) J. Biol. Chem. 271:23992-23998). Because of the inherent flexibility of the linear peptide sequence, inhibition was weak in *in vitro* activity assays. We have recently designed two novel cyclic peptides (Church, W.B. et al.), cFLSYR and a cyclic peptide where F and Y are substituted with 2-naphthylalanine (c(2NapA)LS(2NapA)R). Both have shown

significant improvement in potency over linear peptides. The potent stimulatory effect of exogenous $sPLA_2$-IIA on prostate cancer cell number was completely blocked by the $sPLA_2$-IIA inhibitor, cFLSYR (Fig. 2B) at all concentrations tested.

We next used flow cytometric analysis to determine how $sPLA_2$-IIA affects the distribution of LNCaP cells in different phases of the cell cycle. In $sPLA_2$-IIA containing medium, the proportion of LNCaP cells in the G1 phase decreased from 74% to 62% with corresponding increase of cells in G2/M phase in comparison to untreated cells (Fig. 2C). In the presence of both $sPLA_2$-IIA and its inhibitor (cFLSYR), the proportion of cells in G1 and G2 phase returned to basal levels (Fig. 2C). These results establish the biological importance of the $sPLA_2$-IIA enzyme activity in prostate cancer cells and demonstrate that $sPLA_2$-IIA-induced cell growth can be attributed at least partly to an increased proportion of cells entering G2/M phase from G1 phase.

Example 3: Oncogenic Action of $sPLA_2$-IIA requires $cPLA_2$-α activity.

Currently, two models are proposed to explain the action of $sPLA_2$ on eicosanoid production (Murakami, M., Kudo, I. (2002) J. Biochem. 131:285-292). One is the direct cleavage of membrane phospholipids. In this model, $sPLA_2$ binds directly to plasma membrane phospholipids and the released arachidonic acid serves as a substrate to produce eicosanoids. Lysophospholipids and/or eicosanoid products of arachidonic acid metabolism mediate indirect activation of the endogenous $cPLA_2$-α *via* mobilisation of calcium. The enhanced $cPLA_2$-α activity can in turn result in enhanced production of eicosanoids. The second model is indirect modulation of intracellular eicosanoid pathways *via* cell surface GPI-linked heparan sulphate proteoglycan receptors. The internalised $sPLA_2$ supplies arachidonic acid to downstream enzymes either directly, or indirectly via activation of $cPLA_2$-α through mitogen activated protein (MAP) kinase-mediated phosphorylation.

To determine if $sPLA_2$-IIA-induced cell proliferation depends on $cPLA_2$-α, we treated LNCaP cells with various doses of selective $cPLA_2$ inhibitor, pyrrolidine-1 (Ghomashchi, F. et al. (2001) Biochim. Biophys. Acta Biomembranes 2:160-166) with or without a constant effective dose of $sPLA_2$-IIA (1 nM). Blockade of $cPLA_2$-α abolishes $sPLA_2$-IIA-induced cell growth completely (Fig. 3), demonstrating that $cPLA_2$-α activation is necessary for $sPLA_2$-IIA-dependent cell proliferation. In the absence of $sPLA_2$-IIA, the $cPLA_2$-α inhibitor had no effect on cell growth, suggesting that $cPLA_2$-α does not promote cell proliferation independently in LNCaP cells (see legend Fig. 3).

Example 4: $sPLA_2$-IIA Inhibitors Suppress Endogenous Proliferation.

Based on our finding that the growth-promoting $sPLA_2$-IIA is constitutively expressed in AIPC we have considered the potential of $sPLA_2$-IIA as a target for treatment of AIPC. We reason that a better outcome can be achieved with the PLA_2 inhibitor than with a COX inhibitor alone because the latter suppresses the production of prostaglandins only.

To test the effect of blocking endogenous $sPLA_2$-IIA on cell growth, we firstly determined the basal mRNA levels of $sPLA_2$-IIA in 3 human prostate cancer cell lines. The androgen-independent cell lines PC-3 and DU145 cells were included in the study to also exclude a possible general toxicity of the inhibitors. mRNA encoding $sPLA_2$-IIA was undetectable in DU-145 compared with LNCaP and PC-3 cells (Fig 4A). We then tested the effect of individual inhibitors cFLSYR and c(2Nap)LS(2Nap)R on cell growth over a range of doses (1-100 nM). The proliferation of LNCaP and PC-3 cells was significantly decreased, and the smallest effective dose was 1 nM for both inhibitors (Fig. 4B). In contrast, neither of the inhibitors had an effect on DU145, presumably due to the lack of endogenous $sPLA_2$-IIA. The low but effective dosage and its

specificity for cell lines containing endogenous $sPLA_2$-IIA only, indicate that non-specific cell toxicity is not likely to explain the inhibitory effect.

In summary, the normally androgen-induced $sPLA_2$–IIA gene expression is constitutively activated in androgen-independent prostate cancer. Exogenously added $sPLA_2$–IIA promotes prostate cell proliferation through its enzyme product and $cPLA_2$. A better therapeutic outcome is likely to be achieved with an $sPLA_2$ inhibitor in prostate cancer including the form of AIPC.

All publications mentioned in the above specification are herein incorporated in their entirety by reference. Various modifications and variations of the described methods and system of the invention will be apparent to those skilled in the art without departing from the scope and spirit of the invention. Although the invention has been described in connection with specific preferred embodiments, it should be understood that the invention as claimed should not be unduly limited to such specific embodiments. Indeed, various modifications of the described modes for carrying out the invention which are apparent to those skilled in molecular biology or related fields are intended to be within the scope of the invention.